**Research Article****Jute endophyte *Grammothele lineata* SDL-CO-2015-1 produces an anticancer compound epothilone B**

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**ARTICLE INFO****Article History**

Received: 27 March 2023

Revised: 28 May 2023

Accepted: 11 June 2023

**Keywords:** *Grammothele lineata*, Endophyte, Epothilone B, Anti-Cancer drug, NaPDoS

**ABSTRACT**

Jute (*Corchorus olitorius*) endophytic fungi *Grammothele lineata* SDL-CO-2015-1 was found to produce epothilone B, a well-reported anticancer compound. The genome and RNA sequence data of *G. lineata* revealed the presence of epothilone gene clusters. *In silico* mining using Natural Product Domain Seeker (NaPDoS) identified four genes matching the epothilone gene cluster (Epo D). Epothilone was extracted from the extracellular extract of this fungus using XAD-16 resin. Different analytical techniques, including Thin Layer Chromatography (TLC), Reverse Phase High-Performance Liquid Chromatography (RP-HPLC), and Mass Spectrometry (MS), were used to identify and characterize epothilone following its extraction. TLC bands were visible at 365 nm for both standard epothilone B and samples with identical  $R_f$  values. Extracted epothilone bands from the TLC plate were further purified using RP-HPLC. Using a linear gradient system with methanol and formic acid, LC-MS spectroscopy was done with RP-HPLC purified fraction. The monoisotopic mass of epothilone ( $M+H^+$ ) = 508.10 Da was present in the mass chromatogram. The product ions of 508.10 Da were found with the  $m/z$  values at 320.20 Da and 420.20 Da, the typical  $MS_2$  fragments for standard epothilone B. This finding suggests that the fungal endophyte, *G. lineata* can be a potential source of epothilone B.

**Introduction**

Endophytes are microorganisms that can reside within the plant tissues without causing any harm to the host. In the past few decades, plant scientists have discovered that plants may serve as a reservoir for endless endophytes (Stone et al., 2000). It is generally hypothesized that endophytic bacteria and fungi may produce bioactive compounds and secondary metabolites similar to those made by the host plants (Strobel, 2003). Isolation of endophytes and studying their natural products is now a ubiquitous scientific approach (Schulz et al.,

2002). Because it is generally assumed that the endophytes produce antimicrobial compounds which protect the host plants, some of these compounds are of high medicinal interest because of their antifungal, antibacterial, antimalarial, and other biological activities.

Endophytes gained enormous attention when the detection of taxol in the endophytic fungus (*Taxomyces andreanae*) isolated from the yew plant (*Taxus brevifolia*) was reported at first (Gangadevi & Muthumary, 2008). Taxol was also

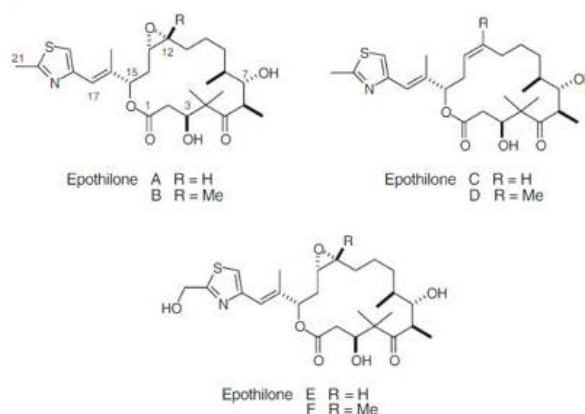
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isolated from Southern Chinese yew (*Taxus mairei*) in the Fujian province of Southeastern China (Wang et al., 2000). In addition, at least three endophytes of *Taxus wallichiana* produce taxol, including *Sporormia minima* and *Trichothecium* spp (Shrestha et al., 2001).

Although an annual dicotyledonous crop, jute (*Corchorus* spp.) is known mostly for its high-quality tensile natural fiber, *C. olitorius* has long been recognized as a medicinal herb, and its extract is known to have apoptotic activity on tumor cell lines (Li et al., 2012). In addition, *C. olitorius* has also been described to possess promising antibacterial and antifungal activity. In the Molecular Biology Laboratory, Department of Biochemistry and Molecular Biology, University of Dhaka, many endophytic fungi were isolated from different parts like roots, stems, leaves, flowers, and fruits of a jute plant (*Corchorus olitorius* var. O-9897) (Zaman, 2019), later they were sequenced and analyzed to see whether they contained any beneficial gene clusters or not. *G. lineata*, a basidiomycete fungus, was an endophyte isolated from jute seedlings, later sequenced, and reported producing Taxol (Das et al., 2017). Some findings suggested another taxol-like substance named epothilone in *G. lineata* extract. Following the discovery of epothilone gene clusters in the genome sequence and RNA-Seq data of *G. lineata*, four genes matching the epothilone gene cluster (Epo D) were identified using NaPDoS (Natural Product Domain Seeker) mining tool.

Epothilones are 16-membered macrocyclic lactones first identified in 1996 (Höfle et al., 1996). The critical structure of epothilones is the macrolide ring with a thiazole-containing side chain and a ketone (Lee et al., 2008). As of September 2008, epothilones A to F have been identified and characterized. Based on the presence or lack of an epoxide group at the C-12 to C-13 position of the macrolide ring, naturally occurring epothilones are categorized as either epoxides (epothilones A, B, E, and F) or olefins (epothilones C and D) (Fig. 1) (Lee et al., 2008).



**Fig. 1. Chemical structure of Epothilone A-F (Lee et al., 2008)**

Epothilone has great commercial value. Such as ixabepilone, which is a second-generation epothilone B derivative and where an azide group had replaced oxygen on position 16 of the macrolide ring, has been approved for injection in combination with capecitabine against metastatic or locally advanced breast cancer resistant-to-taxane by FDA, in 2007 (Conlin et al. 2007; Nicolaou et al. 1997).

In this study, purification and characterization of epothilone in the crude extract of *G. lineata* were performed. *G. lineata* was grown in liquid media to extract epothilone, and after 21 days of growth, epothilone was extracted using well-established procedures. Chemical characterization included spectroscopic analyses such as TLC, HPLC, and LC-MS. This endophyte SDL-CO-2015-1, identified as *G. lineata* is the first-ever Basidiomycete found to possess a capacity for epothilone production.

## Materials and Methods

### Sequencing of *G. lineata*

*G. lineata* SDL-CO-2015-1 isolated from jute seedlings in the Molecular Biology laboratory, Dept. of Biochemistry and Molecular Biology, University of Dhaka, was sequenced and submitted to NCBI (accession number NDF02000000) (Das et al., 2017).

### *In silico* identification of epothilone in *G. lineata*

Natural product database-based screening was done for the sequence of *G. lineata* SDL-CO-2015-1 to find the potential of this fungus to produce important

metabolites. Initially, screening was done with NaPDoS. NaPDoS is a bioinformatic tool that detects and analyzes secondary metabolite genes (Ziemert et al., 2012). This tool detects and extracts condensation (C-) and ketosynthase (KS-) domains from DNA or amino acid sequence data, including PCR amplicon products, individual genes, whole genomes, and metagenomic data sets. Choosing KS as domain type and predicted protein sequences (amino acid) as query type, protein sequence data of *G. lineata* SDL-CO-2015-1 was entered to identify candidate KS domains for significant secondary metabolite production. BLASTx was done to check whether these four genes found by NaPDoS can also be found in the RNA-Seq data. NaPDoS prediction of *G. lineata* producing epothilone was based on the presence of four genes mentioned above that matched with EposD of *S. cellulosum*. We further analyzed the similarity of these proteins with EpoD.

### ***In vitro* identification of Epothilone**

#### **Culture of the *G. lineata***

*G. lineata* SDL-CO-2015-1 was grown in potato dextrose agar (PDA) media to maintain regular subculture and later in potato dextrose broth (PDB) media to obtain the fungal extract and to examine if *G. lineata* produced epothilone. For the submerged culture, the fungus was allowed to grow in an Erlenmeyer flask (500 mL) containing PDB medium (potato 200 g/L and glucose 20 g/L) (Fig. 2).



**Fig. 2. Cell culture images of *G. lineata*. (A) Solid culture of *G. lineata* (left, front, and right, back view). (B) Submerged culture of *G. lineata* in PDB media to obtain fungal extracts. Twenty-one days**

#### **old submerged culture (left) and 10 days old submerged culture (right).**

The overall pH was adjusted to 5.5-5.6, which was optimal for the growth of basidiomycete. Following the inoculation, the flask was incubated at  $\sim 28^{\circ}\text{C}$  and 180 rpm for 21 days in an incubating shaker (LabTech, Model LSI-1005R).

#### **Extraction of epothilone from culture supernatant using XAD-16**

After 21 days of culture, the fungus was filtered to obtain the extracellular components. Firstly, the mycelia were separated from the broth using a gauze bandage in the funnel. Then the supernatant was filtered using filter paper (Whatman® Schleicher and Schuell Ashless filter paper, 110cm). XAD-16 resin extracted epothilone from the supernatant (Cao et al., 2018). 1.5% (w/v) XAD-16 resin was weighed, activated, and shaken with 200 mL filtered culture broth in an Erlenmeyer flask (500 mL). Shaking allowed proper agitation, so XAD-16 could absorb epothilone and its related compounds.

The resins were rescued and dried after 2-3 days of continuous shaking. Dried resins were eventually soaked into carbon tetrachloride where the resin-tetrachloromethane ratio was maintained at 1:4 (w/v). After soaking for 2-3 hours, filtration was carried out using a small-sized column with glass wool placed at the bottom to restrict the flow of resins. Residues were extracted in the same way after filtering 2-3 times. Finally, after combining all extracts, they were evaporated under a vacuum at  $45^{\circ}\text{C}$  in a 25 mL round bottom flask using a rotary evaporator. After complete evaporation, the residues attached to the bottom of the flask were dissolved in 2 mL of HPLC-grade methanol and collected. The samples were then preserved at  $-80^{\circ}\text{C}$  until their subsequent use.

#### **Chromatographic separation of epothilone crude extract**

To detect the presence of epothilone, thin layer chromatographic (TLC) analysis was carried out on

Macherey Nagel & Co. KG 0.2 mm (20×20 cm) silica gel pre-coated plate where petroleum ether: acetone (5:1, v/v) was used as the mobile phase. Samples were loaded at one end, 1 cm from the edge, along with the standard (commercial Epothilone B, Sigma-Aldrich). The TLC bands were then visualized under UV light at a wavelength of 365 nm. A reverse phase C18 column (Acclaim™ 120, C18, 250×4.6 mm, particle size 5µm, pore size 120 Å) was used to analyze the crude extract by RP-HPLC. Standard epothilone and lyophilized epothilone extract were dissolved in HPLC-grade methanol, filtered, and injected into the column at 20 µL. A multistep gradient system of fifty minutes was performed with methanol and nano-pure water with 0.05% TFA (Tri-Fluoro Acetic Acid) with an optimized protocol (retention time 0-10 min, H<sub>2</sub>O+0.05% TFA combination 90%, methanol 10%, retention time 10-30 min, H<sub>2</sub>O+0.05% TFA combination 40%, methanol 60%, retention time 30-40 min, H<sub>2</sub>O+0.05% TFA combination 40%, methanol 60% and retention time 40-50 min, H<sub>2</sub>O+0.05% TFA 90%, methanol 10%). The fraction with the same retention time as the standard was collected. 249 nm wavelength was used to detect compounds eluted from the column. The LC-MS measurement was performed on an Agilent 6400 series Triple Quadrupole B.09.00 utilizing a Waters Acquity BEH C-18 column (100 × 2.1 mm, 2.2-µm particle size). Separation of the sample was obtained with a multistep gradient of A (methanol + 0.1% formic acid) and B (water) at a flow rate of 0.2 mL/min at 25°C. UV spectra were recorded by a DAD (Diode Array Detector) at 254 nm and 249 nm. MS measurement was carried out using the standard ESI (Electrospray Ionization) source that was equipped with a turbo ion spray source operating at 350°C with the fragmentation voltage set to 135V and collision energy at 35V. Mass spectra were acquired in centroid mode

ranging from 100-600 m/z in positive ionization mode with auto MS2 fragmentation.

## Results

### Morphological Characterization of PCR Positive Fungal Isolate, SDL-CO-2015-1

The SDL-CO-2015-1 colonies are white and cottony type, and on the reverse side, they are yellowish-white (Fig. 2), widely effused, growing over the edge of the petri dish and becoming dark with age. No color change is observed when it ~~became~~ becomes dry. The texture of the intermediate layer is highly gelatinized.

### *In silico* identification of epothilone

#### Screening of *G. lineata* sequence based on natural product database

NaPDoS identified four protein sequences from the *G. lineata* sequence data that matched with EpoD of the epothilone gene cluster (Table 1). Notably, the EpoD (EpoD\_Q9L8C7\_4mod) gene of the epothilone gene cluster is crucial in the epothilone production pathway (Fig. 3) (Hardt et al., 2001).

#### Identification of NaPDoS predicted epoD gene product in RNA-seq data

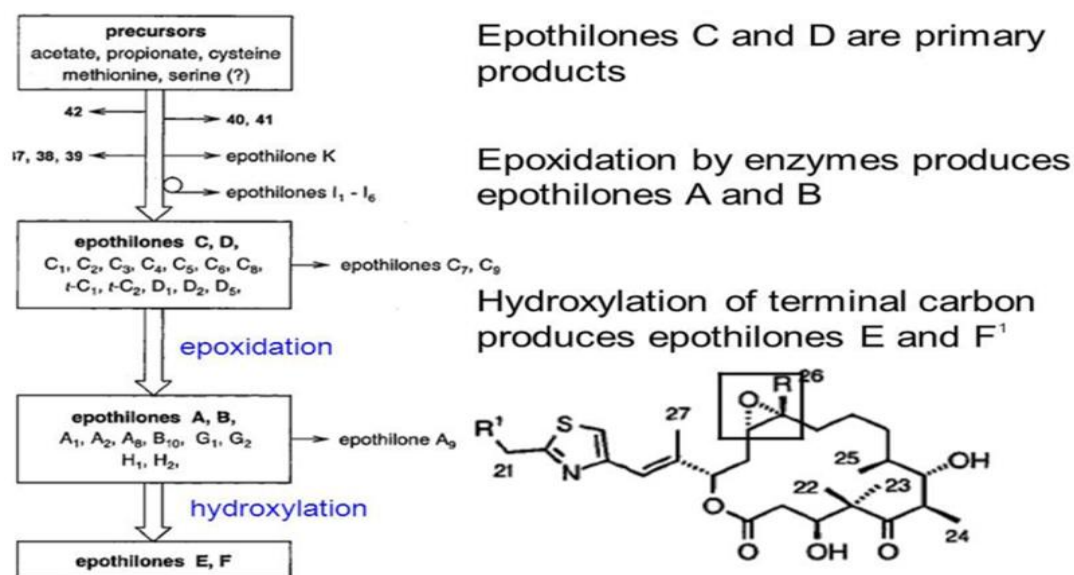
In BLASTx, three out of four genes identified by NaPDoS matched 100% with different genes from RNA-seq data (Table 2).

They are epo2 (>maker-jcf7180000002626-augustus-gene-0.115-mRNA-1 protein AED:0.06 eAED:0.06 QI:0|1|1|1|1|17|0|1935), epo3 (>maker-jcf7180000002765-augustus-gene-0.65-mRNA-1 protein AED:0.06 eAED:0.06 QI:0|1|1|1|1|17|0|1955) and epo4 (>maker-jcf7180000002765-snap-gene-0.98-mRNA-1 protein AED:0.07 eAED:0.07 QI:0|1|1|1|0.94|0.88|18|0|1914) which showed 100% similarity with the genes from RNA-Seq data.

**Table 1. *In silico* identification of epothilone gene cluster in *G. lineata* by NaPDoS**

Query ID	Database Match ID*	E-value	Pathway Product
maker-jcf7180000002760-snap-gene-0.98-mRNA-1	EpoD_Q9L8C7_4mod	6e <sup>-64</sup>	epothilone
maker-jcf7180000002626-augustus-gene-0.115-mRNA-1	EpoD_Q9L8C7_4mod	7e <sup>-57</sup>	epothilone
maker-jcf7180000002765-augustus-gene-0.65-mRNA-1	EpoD_Q9L8C7_4mod	3e <sup>-67</sup>	epothilone
maker-jcf7180000002765-snap-gene-0.98-mRNA-1	EpoD_Q9L8C7_4mod	3e <sup>-54</sup>	epothilone

\*From the *G. lineata* sequencing data, NaPDoS discovered four protein sequences that matched EpoD (EpoD\_Q9L8C7\_4mod) of the epothilone gene cluster.



**Fig. 3. Biosynthesis of Epothilones (Hardt et al., 2001)**

**Table 2. Identification of epothilone-producing gene cluster in *G. lineata* RNA analysis**

Query	Subject	Identity	Alignment	q_start	q_end	s_start	s_end
<sup>a</sup> maker-jcf7180000002626-augustus-gene-0.115-mRNA-1	epo2	100	1935	1	5805	1	1935
<sup>b</sup> maker-jcf7180000002765-augustus-gene-0.65-mRNA-1	epo3	100	1955	1	5865	1	1955
<sup>c</sup> maker-jcf7180000002765-snap-gene-0.98-mRNA-1	epo4	100	1914	1	5742	1	1914

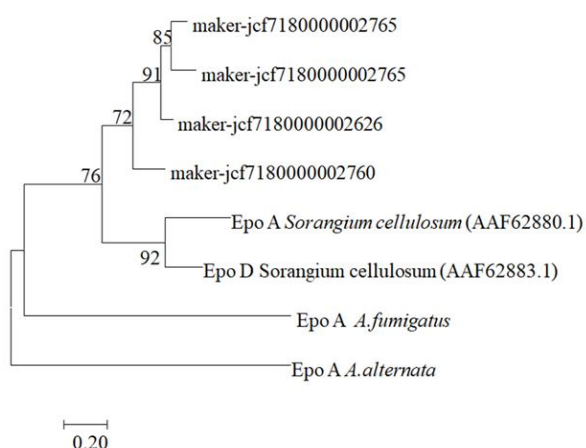
\*In the BLASTx analysis, three out of the four genes identified by NaPDoS showed a 100% match with different genes from RNA-seq data. These genes are <sup>a</sup>epo2, <sup>b</sup>epo3, and <sup>c</sup>epo4.



### Molecular and phylogenetic analysis of epothilone genes

Based on the existence of the four genes listed above that corresponded with EposD of *S.cellulosum*, NaPDoS predicted that *G. lineata* would produce epothilone. Therefore, we further approached to examine how closely these proteins resemble EpoD. BLAST result showed around 35% similarities for all four proteins with EpoD. This result is reasonable as *S. cellulosum* is a bacterium, whereas *G. lineata* is a fungus. So, epothilone must be produced in different pathways in these two microorganisms. However, recently *Aspergillus fumigatus* has been reported to produce epothilone B.

Since it is a fungus, we tried to see if our four protein sequences identified by NaPDoS had better similarity with the EpoA of *A. fumigatus* than with *S. cellulosum*. A phylogenetic tree was constructed using four matched proteins: *A. fumigatus* EpoA, *A. alternata* EpoA, *S. cellulosum* EpoA, and EpoD. *A. fumigatus* EpoA showed the best similarities with the four matched proteins. Four matched proteins had 77 percent similarity with the *A. fumigatus* EpoA (Fig. 4). Moreover, a BLAST search of the four matched proteins in the NCBI database found different polyketide synthase (PKS) gene clusters from different basidiomycete fungi. As the epothilone gene cluster is present in PKS or NRPS, it suggested the involvement of four matched proteins in epothilone production. With all these bioinformatics data, we stepped forward to extract epothilone from *G. lineata*.

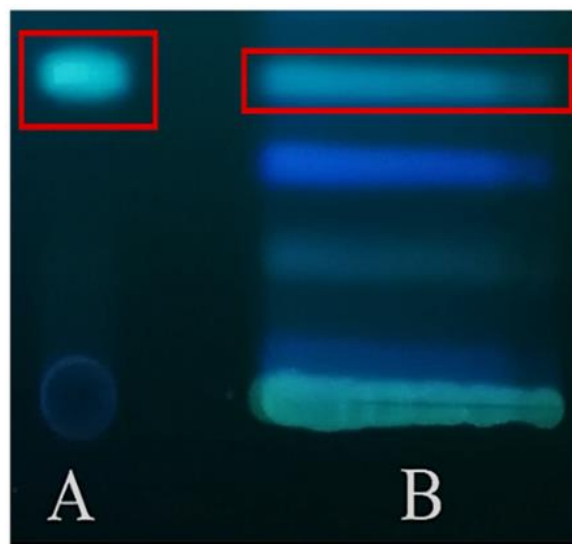


**Fig. 4. Phylogenetic analysis of Epothilone genes cluster. The phylogenetic tree was designed based on genome sequences of *A. fumigatus*, *G. lineata*, *A. alternata*, and *S. cellulosum*.**

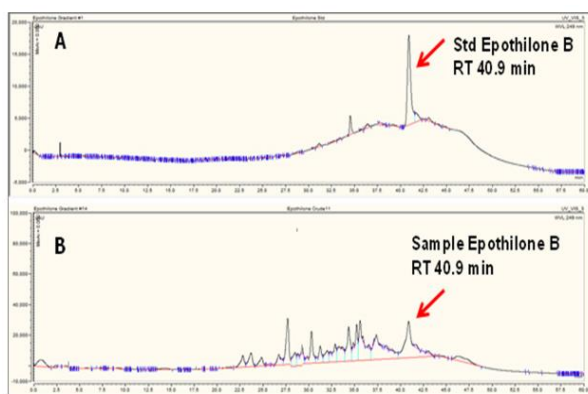
### In vitro identification of Epothilone

#### Chromatographic Separation of *G. lineata* Extract

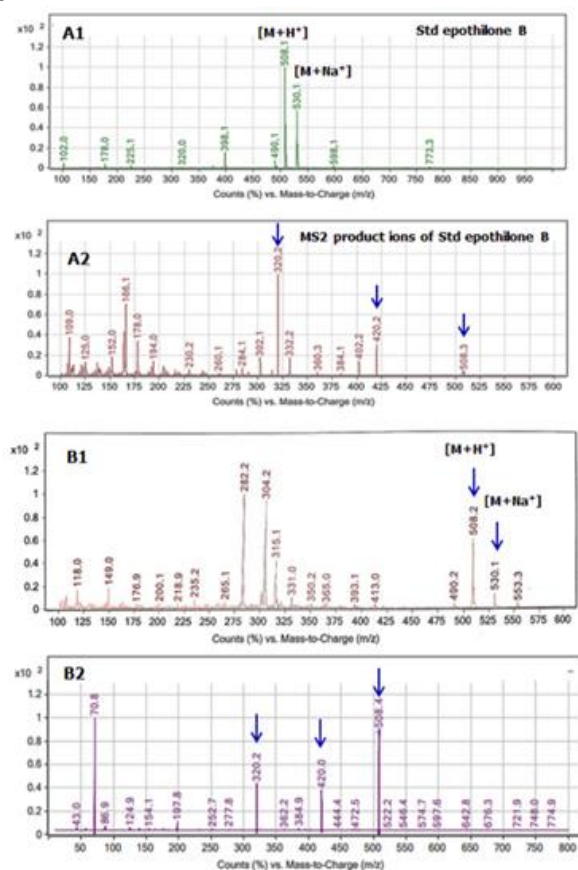
TLC plate showed matching bands of bluish-green color for standard epothilone B and the fungal extract obtained from XAD-16 resin at the UV wavelength of 365 nm (Fig. 5).  $R_f$  values for both these bands were 0.78, which was similar to the previously reported value for epothilone B under the same solvent system of petroleum ether: acetone (5:1, v/v). Standard epothilone B was eluted at a retention time of 40.9 minutes. This peak generated at the 41<sup>st</sup> minute produced a typical spectrum for epothilone B (Yang et al., 2015). A characteristic peak with an almost similar retention time as standard epothilone B was obtained for the extracellular crude extract of the fungus in RP-HPLC, in a multistep gradient solvent system (Fig. 6). The reference epothilone B and the HPLC sample of the SDL-CO-2015-1 extract both produced a distinctive molecular ion peak ( $M+H^+$ ) at 508.1 Da during the LC-MS/MS scan. They also had the typical MS2 fragments at  $m/z$  420.0 Da and 320.2 Da in the production mode (Fig. 7).



**Fig. 5. Putative epothilone B was visualized under UV at 365 nm in TLC plate. (A) The Standard Epothilone B as a control and (B) Bands for the Fungal Extract was seen in the experimental samples performed in this study.**



**Fig. 6. Active fractions images of RP-HPLC column chromatography. (A) Standard epothilone B was eluted at 40.9 minutes. (B) Crude extract from *G. lineata* runs under the same condition generating a peak at 40.9 minutes.**



**Fig. 7. HPLC fractions of *G. lineata* crude extract. Several strong peaks were found in each mass/charge (m/z) state. (A1) Molecular mass [M+H<sup>+</sup>] 508.10, [(M+Na<sup>+</sup>) 530.10] of standard epothilone B and (A2) its MS2 fragments (m/z 420.20 and 320.20) (top right). (B1) Molecular mass [M+H<sup>+</sup>] 508.20, [(M+Na<sup>+</sup>) 530.10, and (B2) MS2 fragments (m/z 420.00 and 320.20) of the 40.9 minute.**

## Discussion

*G. lineata* SDL-CO-2015-1 is an endophytic fungus that had been isolated from jute. It is a basidiomycete fungus and has been reported to produce paclitaxel. The draft genome of this potential fungus sequence was declared in 2017 (Das et al., 2017). During different screening techniques both *in silico* and *in vitro*, there were indications of *G. lineata* SDL-CO-2015-1 producing another important compound named epothilone. These indications led us to work further to extract and identify epothilone from this fungus.

NaPDoS predicted that *G. lineata* SDL-CO-2015-1 might produce epothilone. It was predicted that four endophytic fungus genes showed similarities with the EposD gene of *S. cellulosum*. EposD comprises two of the eight PKS modules (module 6 and module 7) of the epothilone gene cluster in this bacterium. One vital factor to be noted is that the synthesis of the gem-dimethyl unit in epothilone is done by module seven from EPOS D. This gem-dimethyl formation is a striking feature in epothilone production (Molnár et al., 2000). So, the similarity of *G. lineata* with EposD increases the chances of epothilone being produced by this fungus.

The jute endophyte *G. lineata* SDL-CO-2015-1 was found to be able to produce epothilone B in our investigation. This assertion was verified by various *in silico* and *in vitro* analytical techniques, although the whole process took a very long time. Firstly, from the *G. lineata* sequencing data, NaPDoS discovered four protein sequences that matched EpoD of the epothilone gene cluster. To determine whether these four genes discovered by NaPDoS can also be found in the RNA-Seq data, another *in silico* approach BLASTx, was used. In BLASTx results, three of the four genes, epo2, epo3, and epo4, were also found to have 100% similarity to other genes in the RNA-seq data. Molecular and phylogenetic analysis of epothilone genes also supported it. In the case of *in vitro* analysis, TLC, RP-HPLC, and LC-MS/MS were performed to confirm the presence of epothilone in the sample. TLC plate presented bands of the same color

and  $R_f$  value for the standard and the purified epothilones from the endophyte culture.  $R_f$  values for each of these bands were 0.78, comparable to the value for epothilone B in the same petroleum ether: acetone solvent system that was previously reported. The standard epothilone B and the purified epothilones from the endophyte culture gave a characteristic peak at the same retention time, 40.9 minute. Finally, LC-MS data identified epothilone B's molecular mass and MS2 fragments.

*G. lineata* has not been reported to produce epothilone in any literature. It is not only the first fungus but also the first eukaryote to produce epothilone. Epothilone is produced by only a few strains of the soil myxobacterium, *Sorangium* (S. guang Li et al., 2014). No other myxobacterium, and indeed no other organism, has been found to produce epothilone so far. So, *G. lineata* is the second organism after *Sorangium* to produce this valuable compound. The discovery of the first-ever epothilone-producing Basidiomycota fungus is another significant finding. Fungi under the phylum of Basidiomycota are known to produce a variety of cytotoxic compounds (Sandargo et al., 2019). Endophytic fungi that produce anticancer components have been described up to this point in time in roughly 46 genera and 111 species. Taxonomically, 96% of them are Ascomycota, with just 3% of them being from the Basidiomycota phylum (Chen et al., 2016) Thus, *G. lineata* with the ability to produce epothilone may not be surprising under these circumstances. The largely untapped fungal kingdom may reveal more members capable of producing diverse bioactive compounds.

Epothilone has been reported to have narrow antifungal activity (Hofle et al., 1996). Since the epothilone-producing fungus, *G. lineata* is an endophyte; it may be assumed that epothilone production may have a role in providing an extracellular barrier against pathogens (fungicidal) to its host plant, or it may be produced to defeat other fungi as a survival instinct.

## Conclusion

Only a few strains of myxobacterium produce epothilone, *Sorangium* has reported so far. Due to the limited production of this expensive compound from microbial origin, there are no options other than chemical synthesis for large-scale production. In this study, we report that a novel fungi *G. lineata* SDL-CO-2015-1, isolated in our lab, can produce epothilone B in culture conditions. The culture condition and production is yet to be optimized for scale-up. Moreover, strain improvement can be done using mutational analysis. Cytotoxicity tests in the cancer cell line must be performed using purified epothilone.

## Conflict of Interest

The authors declare no competing financial interest.

## Acknowledgments

The project is funded by ICGEB, Trieste, Italy (CRP/BGD17-01). The authors thank the Bangladesh Council for Scientific and Industrial Research (BCSIR) for their LC/MS support.

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